ORIGINAL ARTICLE

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Capacity of Omega-3 Fatty Acids or Eicosapentaenoic Acid to Counteract Weightlessness-Induced Bone Loss by Inhibiting NF-κB Activation: From Cells to Bed Rest to Astronauts

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ABSTRACT

NF-κB is a transcriptional activator of many genes, including some that lead to muscle atrophy and bone resorption—significant concerns for astronauts. NF-κB activation is inhibited by eicosapentaenoic acid (EPA), but the influence of this omega-3 fatty acid on the effects of weightlessness are unknown. We report here cellular, ground analogue, and spaceflight findings. We investigated the effects of EPA on differentiation of RAW264.7 monocyte/macrophage cells induced by receptor activator of NF-κB ligand (RANKL) and on activation of NF-κB by tumor necrosis factor α (TNF-α) or exposure to modeled weightlessness. EPA (50 μM for 24 hours) inhibited RANKL-induced differentiation and decreased activation of NF-κB induced by 0.2 μg/mL of TNF-α for 30 minutes or by modeled weightlessness for 24 hours ($p < .05$). In human studies, we evaluated whether NF-κB activation was altered after short-duration spaceflight and determined the relationship between intake of omega-3 fatty acids and markers of bone resorption during bed rest and the relationship between fish intake and bone mineral density after long-duration spaceflight. NF-κB was elevated in crew members after short-duration spaceflight, and higher consumption of fish (a rich source of omega-3 fatty acids) was associated with reduced loss of bone mineral density after flight ($p < .05$). Also supporting the cell study findings, a higher intake of omega-3 fatty acids was associated with less *N*-telopeptide excretion during bed rest (Pearson $r = –0.62$, $p < .05$). Together these data provide mechanistic cellular and preliminary human evidence of the potential for EPA to counteract bone loss associated with spaceflight. © 2010 American Society for Bone and Mineral Research. 2010 ASBMR

KEY WORDS: RECEPTOR ACTIVATOR OF NUCLEAR FACTOR-κB; OSTEOCLASTS; EICOSAPENTAENOIC ACID; WEIGHTLESSNESS; WEIGHTLESSNESS SIMULATION

Introduction

Adaptations to weightlessness and associated health risks of spaceflight are extensive. They include alterations in the musculoskeletal, cardiovascular, neurovestibular, immune, endocrine, and other physiologic systems. Understanding the nature and mechanisms of changes in human physiology during space travel and developing means to counteract these effects where necessary are primary concerns for the National Aeronautics and Space Administration (NASA) and the space agencies of other nations.

Muscle catabolism and bone loss are two physiologic changes that occur on exposure to weightlessness, and both could degrade health and performance during a mission and on return to gravity.[1–3] There is great interest in developing countermeasures to mitigate these processes, and although exercise (and other loading) and pharmacologic countermeasures have been proposed and tested, success has been limited. A dietary countermeasure would be ideal to minimize launch mass, crew time and inconvenience, and risk of side effects.

The activation of nuclear factor-κB (NF-κB) is associated with the induction of transcription factors critical for many processes related to injury and inflammation, including various etiologies of muscle catabolism and osteoclastogenesis. Hindlimb suspension, a commonly used ground-based animal model of spaceflight, consistently shows evidence for activation

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of NF-κB, which is thought to be associated with the mechanism of muscle atrophy observed in this model.[4–7] In hindlimb unloading models, the activation of NF-κB may result from altered mRNA expression of NF-κB subunits p50, Bcl-3, and possibly c-Rel.[6] Other unloading studies have shown a role for *IκBα* but not c-Rel.[5] Along with increasing expression of NF-κB mRNA, skeletal muscle hindlimb unloading in rats also increases NF-κB activation. Clearly, multiple potential mechanisms can be hypothesized for the increase in NF-κB activation during unloading.

The body of evidence is now substantial that dietary long-chain omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) have beneficial effects on bone[8–11] and on muscle during conditions with increased proteolysis and increased activation of NF-κB.[12–15] The protective effect of EPA has been attributed to its anti-inflammatory actions and inhibition of signaling factors, such as NF-κB, that are associated with inflammation and downstream activation of the ubiquitin-proteasome system and osteoclasts.[14,16–20] In vitro models show that EPA can inhibit activation of NF-κB by several different stimuli, including lipopolysaccharide, RANKL, tumor necrosis factor α (TNF-α), arachidonic acid, and proteolysis-inducing factor.[8,17,21–24]

Evidence that modeled weightlessness can activate NF-κB and that EPA can inhibit activation of NF-κB gives reason to believe that EPA could inhibit this pathway during spaceflight or modeled spaceflight and have a protective effect on bone. In this article we report the results of one study evaluating NF-κB activation in humans before and after short-duration spaceflight and three studies done to investigate the effects of EPA on NF-κB activation or its effects in three different systems. The first system was a cell culture system that models weightlessness. We investigated the effect of EPA on osteoclast differentiation and NF-κB protein expression and activation in this system. To accomplish this, we used a rotary cell culture system (RCCS) equipped with a rotating wall vessel bioreactor.[25,26] In the RCCS, the culture vessel rotates around a horizontal axis, and the cultured cells, bound to microcarrier beads, rotate at the same speed as the vessel. This simulates weightlessness by randomizing the gravitational vector. The second system was bed rest, a ground-based analogue of weightlessness.[2,27] We investigated the relationship between the intake of omega-3 fatty acids and bone-resorption markers in healthy volunteers during 60 days of bed rest. The third system was long-duration spaceflight; we evaluated the relationship between fish (and EPA) intake and bone mineral density (BMD) of astronauts on missions of 4 to 6 months on the International Space Station.

Materials and Methods

Materials

EPA was purchased from Cayman Chemical Company (Ann Arbor, MI, USA), and PBS and α-MEM components were purchased from GIBCO (Invitrogen Corp., Carlsbad, CA, USA). RANKL was from Cell Signaling Technology Company (Danvers, MA, USA), and RAW264.7 murine monocyte/macrophage cells were purchased from the American Type Culture Collection (Manassas, VA, USA).

Cell culture

RAW264.7 cells were maintained in DMEM with 10% sterile-filtered fetal calf serum (FCS) supplemented with 2 mM of glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The cells were grown at 37°C in 5% CO_2 in humidified air.

In vitro generation of osteoclasts and determination of osteoclast phenotype

RAW264.7 cells were plated in the medium just described and incubated in the presence of 50 ng/mL of RANKL for 5 days in T-75 flasks (Fisher Scientific, Pittsburgh, PA) with or without EPA (50 μM). EPA was first dissolved in ethanol as a stock solution and freshly prepared with each experiment by diluting it in growth medium before adding it to the cells. After the fifth day, the cultures were split, and cells were plated on chamber slides with two chambers on each slide (Nunc, Fisher Scientific) at a concentration of 1.5×10^6 cells per well. After the cells reached approximately 80% confluence, the phenotype was determined by staining the cells for tartrate-resistant acid phosphatase (TRACP) activity (Acid-Phosphatase Kit, Sigma-Aldrich). Four slides were prepared for each condition. Differentiated osteoclasts were counted as the number of multinucleated, $TRACP^+$ cells on each slide. Additionally, NF-κB p65 activity and protein expression were determined as described below.

Nuclear extraction

For collection of nuclear proteins from the cells, the cells were first washed with PBS. They were scraped off the culture dish and then collected using a Chemicon nuclear extraction kit according to manufacturer's instructions (Millipore, Billerica, MA, USA).

NF-κB–DNA binding assay

The active form of NF-κB from nuclear fractions was measured using a commercially available ELISA-based kit (StressGen Biotechnologies, Victoria, Canada). Data are expressed as units of activity per microgram of protein.

Western blotting

The concentrations of protein were determined using a Markwell protein assay.[28] The protein in the cell extract was concentrated with 10% trichloroacetic acid (Sigma-Aldrich). Equal amounts of cell extract (200 μg) were resolved with 10% SDS-PAGE (precast Long-Life Gels, VWR, West Chester, PA, USA) using samples diluted in 20 μL of sample dilution buffer (Sigma-Aldrich) as described previously.[29] The resolved gel was equilibrated in cold transfer buffer for 10 minutes and then was electroblotted to polyvinylidene fluoride (PVDF; Immobilon-P, Millipore) for 1 hour at 30 V.[29] The blot was stained with amido black stain [50% (v/v) methanol, 10% (v/v) glacial acetic acid, and naphthol blue black] and destained [50% (v/v) methanol and 10% (v/v) glacial acetic acid] and then washed with two changes of methanol before allowing it to air dry.

For the detection of NF-κB p65, the blot was incubated in 0.5% nonfat dry milk (NFDM) in Tris-buffered saline (20 mmol/L of Tris, pH 7.4, 150 mmol/L of NaCl) with 0.05% (v/v) Tween-20

(TBST) for 1 hour at room temperature. Subsequently, a 1:1000 dilution of rabbit anti-NF-κB p65 (Cell Signaling Technology) in a 0.5% NFDM-TBST solution was incubated with the blot overnight at 4°C. The blot was washed with three changes of TBST without NFDM and then incubated with 1:2000 goat anti-rabbit IgG–horseradish peroxidase (Cell Signaling Technology) for 1 hour at room temperature with gentle shaking. The PVDF was washed three times in TBST without NFDM. A chemiluminescent substrate that exhibits fluorescent properties (ECL-Plus, GE Healthcare Life Sciences, Pittsburgh, PA, USA) then was applied to the blot and allowed to stand for 5 minutes. Placing the blot back into TBST stopped the reaction. NF-κB p65 protein was detected and emitted fluorescence quantified on a Storm fluorescent optical scanner as described by the manufacturer (GE Healthcare Life Sciences). The specificity of this detection was confirmed through competition with excess p65.

Effect of EPA on NF-κB activation induced by TNF-α in differentiated osteoclasts

Differentiated osteoclasts were grown to approximately 70% confluence in T-75 flasks and then incubated for 24 hours with or without 50 or 100 μM of EPA. NF-κB was activated by incubation with TNF-α (0.2 μg/mL) for 30 minutes. The cells from the T-75 flasks were washed with PBS, and nuclear proteins were extracted as described earlier. NF-κB p65 protein expression and activation were determined.

Effect of EPA on NF-κB activation induced by simulated microgravity in differentiated osteoclasts

A high-aspect-ratio vessel (HARV) was used to provide an environment for the cells where they would be in a continuous state of free fall, similar to what is experienced in weightlessness (Synthecon, Houston, TX, USA). The 50-mL bioreactor was inoculated with Cytodex beads (Pharmacia, Piscataway, NJ) with a bead/cell ratio of 1:20; it was completely filled with medium, and all bubbles were removed. The bioreactor was placed in an incubator at 5% CO_2, 99% humidity, and 37°C. The vessel was rotated at a rate that maintained suspension of the bead-cell complexes (about 12 rotations per minute).

A stationary coculture of RAW264.7 cells was grown and seeded onto Cytodex beads at the same time and concentration as the cells placed in the bioreactor. These cells were maintained in T-75 flasks next to the bioreactor in the incubator throughout the studies.

Differentiated osteoclasts in the HARV and stationary coculture systems were grown to approximately 70% confluence and then incubated for 24 hours with or without 50 μM of EPA. After 24 hours, the cells were processed, and NF-κB activation and protein expression were determined as described earlier.

Studies with human subjects

Although the basic findings of the studies with human subjects have been published previously (see details below), the relationships between dietary intake and bone health have not been evaluated. As described in the original publications, all protocols were reviewed and approved by the NASA Johnson Space Center Committee for the Protection of Human Subjects, and informed consent was obtained as required.

Spaceflight: Short duration

We examined NF-κB p65 expression before and after short-duration spaceflight in a study with 10 subjects (7 males, 3 females, aged 36 to 54 years) from four shuttle missions (12 to 16 days each). No crew member participated in more than one of these missions. Crew members were age- and sex-matched with 7 healthy control subjects (5 male and 2 female) who were not involved in spaceflight activities.

Sample Collection and Preparation: Blood samples (10 mL) were collected from the crew members 10 days before launch (L – 10), within 2 to 3 h after landing (R + 0), 14 days after landing (R + 14), and at their postflight annual medical exams (AME) about 3 to 4 months after flight.

Peripheral blood mononuclear cells (PBMCs) were prepared by the method described previously.[30,31] Cells were counted with a hemocytometer and resuspended in 1 mL of freezing medium consisting of 90% pooled human blood group AB serum (BioWhittaker, Walkersville, MD) and 10% dimethyl sulfoxide (Sigma-Aldrich). These cells then were cryopreserved by placing them in a −270°C bath of 100% ethanol and then transferred the next day to liquid nitrogen, where they were stored until processed.

Immunolocalization of NF-κB p65: The nuclear localization of p65 was examined using an immunocytochemical method as described previously.[32]

Spaceflight: Long duration

During long-duration spaceflight, crew members record their dietary intake once per week using a food frequency questionnaire (FFQ).[33,34] A unique FFQ is designed for each crew member based on the foods flown for that mission (NutritionQuest, www.NutritionQuest.com). This FFQ has been validated in a ground-based model of long-duration spaceflight[33] and implemented on both the Russian Mir space station in the 1990s[33] and all International Space Station (ISS) missions to date. Preliminary findings were published in 2005.[34] Given that (1) space food systems are largely closed (because the number of food items is limited and the menu cycle is repetitive), (2) portion sizes are known, and (3) the precise nutrient content is known for each food item in the system, the FFQ designed for spaceflight is more reliable than a standard food questionnaire.

The FFQ is designed to obtain a near-real-time estimate of intakes of seven key nutrients—energy, protein, water, sodium, calcium, iron, and potassium—as well as to collect information about supplement use and any crew comments.[35] In developing each FFQ, available foods are categorized by content of the key nutrients, and crew members are asked how many of each of the food items in that category were consumed that week. Through evaluation of the food item data (as opposed to nutrient data), the average number of fish servings per week was determined, to estimate omega-3 fatty acid intake during flight. Examples of fish selections in the ISS food system are salmon, pike, perch, sturgeon, anchovies, sardines, mackerel, and tuna.

Two 24-hour urine pools were collected from 24 crew members about 6 months and 6 weeks before flight and during

the first 48 hours after landing. *N*-Telopeptide (NTX) was determined in each 24-hour pool, and postflight NTX was expressed as a percent change from the average of all four of the preflight 24-hour pool data points. As described previously,[34] BMD was determined about 180 and 45 days before and 5 days after each flight by dual-energy X-ray absorptiometry (DXA) with a fan-beam densitometer (Hologic QDR 4500W, Hologic, Inc., Waltham, MA, USA). These data, both bone marker and DXA data, have been published previously,[1,34] but without regard to relationship to diet (as included here).

The changes in trochanter BMD and NTX after flight were correlated with the estimated number of fish meals per week to approximate omega-3 fatty acid intake. It was not possible to determine precise omega-3 fatty acid intake from the FFQ data because many of the fish items were categorized together in the FFQ.

Bed rest

Sixteen subjects participated in a series of bed rest studies conducted at the University of Texas Medical Branch General Clinical Research Center (GCRC; Galveston, TX, USA) that were undertaken to evaluate various physiologic and nutritional changes during and after 60 or 90 days of 6-degree head-down-tilt bed rest. Details of the bed rest protocols[36,37] and dietary control[38] have been described elsewhere. Briefly, a research dietitian worked with each subject to come up with a menu to meet all the subject's nutrient requirements and maintain body weight. Some variability in food choice was allowed so that individual preferences and tolerances could be met while specific nutrient intakes were maintained. Although protein and fat (and other nutrients) were controlled, fish intake was not. All food was prepared in a metabolic kitchen, and subjects were required to consume 100% of the food provided and to consume nothing in addition to what was prepared in the kitchen. Diets were controlled for 10 days before bed rest started while the subjects were residing in the GCRC but were able to move around the unit.

All foods were weighed, and actual dietary intakes were determined using the Nutrition Data System for Research (NDS-R) software, Version 5.0-35, May 2004, developed by the Nutrition Coordinating Center, University of Minnesota (Minneapolis, MN, USA).[39] Total omega-3 fatty acid intake was summed, and a mean intake per day was determined.

Twenty-four-hour urine pools were collected 10, 9, 3, and 2 days before bed rest. Twenty-four-hour pools also were collected around days 59 and 60 of bed rest. Aliquots of the combined voids were frozen at -80°C until they were analyzed. NTX concentrations were determined in each of the 24-hour urine pool samples as described previously.[34] The four pre–bed rest values were averaged, and the 59- and 60-day bed rest data were averaged. The percent change (from pre–bed rest) of NTX was determined and correlated with the mean daily omega-3 fatty acid intake during the first 60 days of bed rest. Data from the first 13 subjects are reported. These are considered control subjects for other (ongoing) studies, in that no countermeasures were applied. The NTX data have been published,[37,40] but without regard to relationship to diet (as included here).

Statistical analysis

In the cell studies a *t* test was used to determine whether the means of two groups were significantly different or a one-way ANOVA and post hoc Bonferroni *t* tests were used to determine which of three or more groups were different from each other. A two-way ANOVA was used to determine the effects of the modeled weightlessness and EPA treatment, with a post hoc Bonferroni *t* test to compare groups. Results from the cell studies are representative of at least three separate evaluations.

A two-way repeated-measures ANOVA was used, with flight and time as variables, to determine the effects of flight on NF-κB activation. For this analysis, the AME data from the flight subjects were not included in the model. In a separate analysis, all data from the flight subjects, including the AME data, were compared in a one-way repeated-measures ANOVA to determine whether NF-κB activation was still changed 3 to 4 months after landing.

Pearson correlation coefficients were determined for the association of omega-3 fatty acid intake with markers of bone resorption after bed rest. A *t* test was used to determine whether bone markers differed between crew members who consumed low (fewer than 3 servings per week) and high (more than 3 servings per week) numbers of fish servings per week. A one-way repeated-measures ANOVA was used to determine whether the total omega-3 fatty acid intake during the 60 days of bed rest changed with time. All statistical analyses were performed using SigmaStat 3.1 (SYSTAT Software, Richmond, CA, USA).

Results

Effect of EPA on RAW264.7 differentiation induced by RANKL

The number of $TRACP^{+}$ cells was 57% lower after 5 days of exposure to 50 μM of EPA than after exposure to RANKL alone (13.5 ± 0.6 and 31.3 ± 7.2 $TRACP^{+}$ cells per well, respectively; $p < .05$), indicating that EPA inhibits RANKL-induced differentiation of RAW264.7 cells. RANKL itself did not change the activation of NF-κB after 5 days of exposure, but EPA decreased both activation and protein expression of NF-κB in RANKL-treated cells (Fig. 1).

Effect of EPA on NF-κB activation induced by TNF-α in differentiated osteoclasts

TNF-α increased NF-κB activation nearly 700% ($p < .001$) and protein expression 15% ($p < .05$) after 30 minutes (Fig. 2). EPA decreased the activation and protein expression in a dose-dependent manner when the cells were exposed to it for the 24 hours before TNF-α stimulation (Fig. 2).

Effect of EPA on NF-κB activation induced by simulated microgravity in differentiated osteoclasts

Simulated weightlessness in the rotating HARV alone increased NF-κB activation by 10% ($p < .05$) and protein expression by 26% ($p < .01$) after 24 hours (Fig. 3). EPA decreased NF-κB activation about 25% ($p < .001$) and protein expression about 15% ($p < .01$)

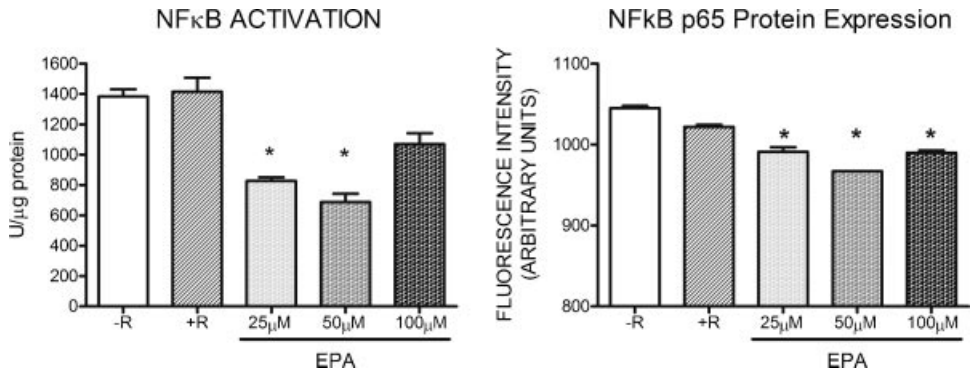

Fig. 1. NF-κB activation and protein expression in RAW264.7 cells after 5 days of exposure to 50 ng/mL of RANKL (±R) with or without (+R) eicosapentaenoic acid (EPA). Symbols indicate significant differences ($p < .001$) from RAW264.7 cells not exposed to RANKL (–R).

in both the stationary coculture and the simulated weightlessness environment.

Short-duration spaceflight

NF-κB p65 expression increased nearly 500% in the shuttle crew members ($p < .001$) after flight (R + 0), and it remained elevated for at least 14 days after flight (Fig. 4). No significant difference was observed between L – 10 and baseline values of the astronauts or between the three values for the control group.

Long-duration spaceflight

The mean number of servings of fish per week during spaceflight was 2.1 ± 1.5 servings per week ($n = 17$), and the number ranged from 0 to 4.6 servings per week. A higher intake of fish was associated with a smaller decrease in whole-body bone mineral density (BMD) after flight (Pearson $r = 0.46$, $p < .05$; Fig. 5). A higher intake of fish tended to decrease the change in whole-body bone mineral content when the latter was adjusted for height ($p = .08$; Fig. 5). The estimated number of fish servings per

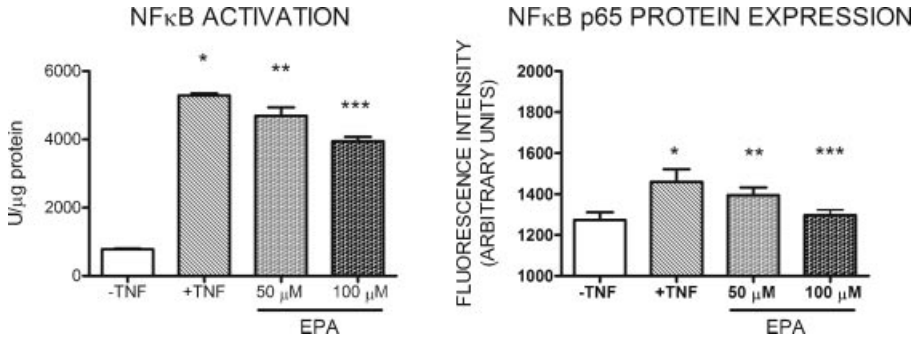

Fig. 2. NF-κB activation and protein expression in differentiated RAW264.7 cells after 30 minutes of exposure to 0.2 μg/mL of TNF-α (+TNF) with or without an earlier 24-hour incubation with eicosapentaenoic acid (EPA). Different symbols indicate statistically significant differences between the effects of different doses. EPA decreased NF-κB activation ($p < .001$) and p65 protein expression ($p < .05$) in a dose-dependent manner.

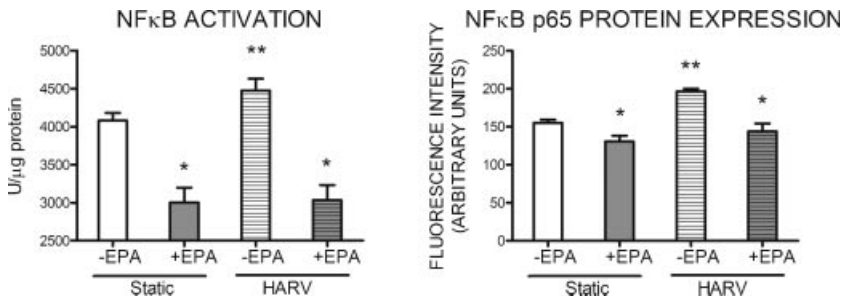

Fig. 3. NF-κB activation and protein expression in differentiated RAW264.7 cells after 24 hours of exposure to modeled weightlessness in a rotating high-aspect-ratio vessel (HARV) or static control with or without an earlier 24-hour incubation with eicosapentaenoic acid (EPA). Different symbols on each graph represent statistically significant differences between groups. The effect of the HARV ($p < .05$) and EPA ($p < .001$) on NF-κB activation and the effect of HARV ($p < .01$) and EPA ($p < .01$) on p65 protein expression were all significant.

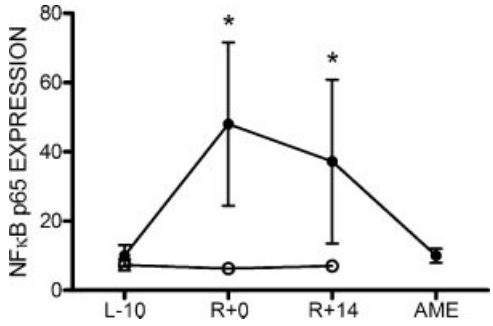

Fig. 4. Expression of NF-κB p65 in peripheral blood mononuclear cells of shuttle crew members ($n = 10$, *filled circles*) and age- and sex-matched nonflight controls ($n = 7$, *open circles*) before flight (L – 10), 3 to 4 hours after landing (R + 0), 14 days after landing (R + 14), and 3 to 4 months after landing (annual medical exam, AME). *Significant difference ($p < .001$) between the flight and control groups at R + 0 and R + 14 and a significant difference from L – 10 for the flight group when a two-way repeated-measures ANOVA was performed (excluding the AME data point). When a one-way repeated-measures ANOVA was performed on the flight data, the R + 0 and R + 14 time points were both different ($p < .001$) from L – 10 and AME, and AME was not different from L – 10.

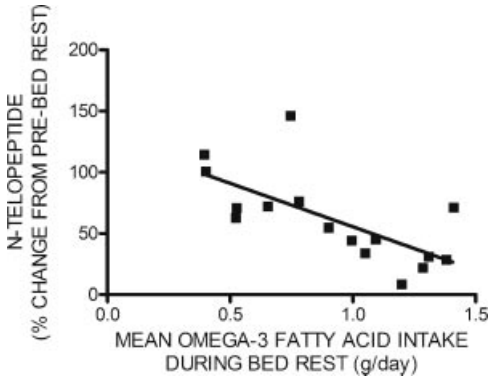

Fig. 6. Correlation between the mean daily intake of total omega-3 fatty acids and the percent change in urinary *N*-telopeptide excretion from pre–bed rest values ($n = 16$, Pearson $r = -0.62$, $p < .01$). The intake of omega-3 fatty acids for each subject is the mean daily intake over the first 60 days of bed rest.

week was not related to the percent change in urinary NTX excretion after spaceflight (data not shown).

Bed rest

The mean intake of total omega-3 fatty acids during 60 days of bed rest was negatively correlated (Pearson $r = -0.62$) with the percent change in urinary NTX excretion from before bed rest to during bed rest ($p < .05$; Fig. 6). Total omega-3 fatty acid intake was not significantly different over the course of the 60 days of bed rest. In Fig. 7, the change in urinary NTX excretion during bed rest and after spaceflight is compared; the overlapping time points were not significantly different.

Discussion

Activation of NF-κB by cytokines including TNF-α has been clearly documented. The data presented here are the first to show that NF-κB is also activated by short-duration spaceflight, and this finding is supported in a ground-based model that exhibits some of the effects of weightlessness on cell cultures, including reduced shear stress. We also present evidence, from spaceflight itself as well as several ground analogues, that suggests that omega-3 fatty acids may have a protective effect on bone and that the mechanism may be related to decreased activation of NF-κB.

By a process that included decreasing NF-κB activation and p65 expression, EPA inhibited differentiation of RAW264.7 cells into osteoclast-like cells. RANKL stimulates RAW264.7 cells to differentiate, but there was no evidence that RANKL activated NF-κB in these studies (note the *y*-axis scale in Fig. 1). It is likely that the activation was transient, occurring before 5 days, as we would have expected RANKL to activate NF-κB. EPA also decreased the NF-κB activation and protein expression induced by exposure to TNF-α for 30 minutes. TNF-α is a known activator of NF-κB, and others have reported that omega-3 fatty acids can inhibit this activation.[41] In cell culture models, simulated weightlessness increases TNF-α production,[42,43] and it activates NF-κB in other cell culture and animal models.[44–47] This is why we predicted that NF-κB would be activated in this model and would be inhibited by EPA.

Exposure to a model that exhibits some of the effects of weightlessness, as observed in the HARV, increased NF-κB activation and p65 protein expression after 24 hours. Further studies need to be performed to determine the time course of activation after exposure to simulated weightlessness because our study shows only one time point. The hindlimb suspension

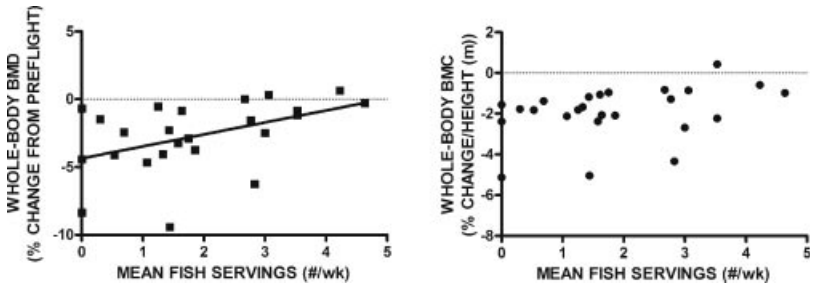

Fig. 5. Correlation of the reported intake of fish servings per week during long-duration spaceflight with the percent change in whole-body BMD after flight (*left*, $n = 24$, Pearson $r = 0.46$, $p < .05$) and with the percent change in whole-body bone mineral content (BMC) corrected for height after flight (*right*, $p = .08$).

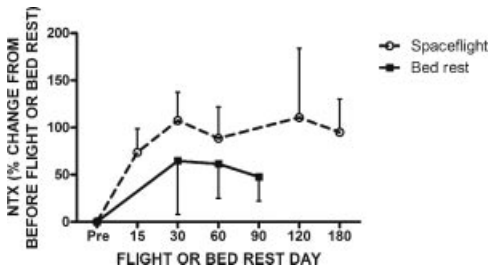

Fig. 7. *N*-telopeptide (NTX) excretion in bed rest and spaceflight expressed as the percent change from before flight or bed rest. $n = 8$ for flight days 15, 30, and 60; $n = 7$ for flight day 120; $n = 4$ for flight day 180; $n = 16$ for bed rest days 30 and 60; and $n = 9$ for bed rest day 90. Pre–bed rest urinary NTX excretion was 452 ± 214 nmol/day (34.1 ± 15.9 nmol/mmol of creatinine), and preflight NTX excretion was 331 ± 135 nmol/day (23.9 ± 9.8 nmol/mmol of creatinine).

model, another ground analogue for weightlessness, is associated with increased expression of an NF-κB-dependent reporter after 7 days, although the p50 and not the p65 subunit was increased.[6] Other studies of unloading have shown a similar activation of NF-κB within several days to weeks, but it involved different mechanisms of activation.[5,48] The increased activation of NF-κB after 12 to 16 days of spaceflight in this study is striking and suggests that the activation is not a transient effect.

The NF-κB pathway is complex and is activated by phosphorylation, ubiquitination, and proteolysis of the inhibitory protein IκB, which nominally binds NF-κB in the cytosol in the inactive form. Activation of the NF-κB pathway in modeled or actual weightlessness could involve a number of different mechanisms, and different subunits such as p50, c-Rel, and Ccl-3 need to be investigated to better understand the whole picture. We chose to first study the effects of EPA on p65 in the HARV model because previous work showed that EPA and docosahexaenoic acid (DHA) could inhibit nuclear p65 expression in RAW264.7 cells.[8]

Clearly, more studies should be done to better characterize the effects observed here, including time-course studies and dose-response effects on differentiation. For example, the reason NF-κB activation in osteoclasts did not decrease even though a decrease in protein expression was observed should be investigated. Further studies need to be done to determine if in fact EPA has a dose-response effect on osteoclast differentiation, perhaps with lower doses than those used in this study.

The bed rest and spaceflight data provide evidence that the amount of omega-3 fatty acids required to observe a protective effect on bone is nutritional and not pharmacologic. If it is assumed that a 100-g serving of salmon provides about 930 mg of total omega-3 fatty acids,[49] then the bed rest subjects consumed the equivalent of 0.5 to 1.5 servings of salmon (or a food item with an equivalent amount of omega-3 fatty acids) per day. Long-duration crew members consumed an overall average of 2.1 fish servings per week (0.3 servings per day, or less than the bed rest subjects consumed).

The lower percent change in whole-body BMD after spaceflight in crew members who consumed more fish meals per week (and the tendency for the lower percent change in whole-body bone mineral content adjusted for height) supports the finding of Rousseau and colleagues that a higher self-reported dietary intake of omega-3 fatty acids is associated with higher BMD in older adults.[50] That study divided the omega-3 intake into a high (>1.27 g/day) and a low group (<1.27 g/day), and if the same assumption applies (a 100-g salmon serving equals 930 mg of total omega-3s), then 1.27 g/day is approximately equal to 1.3 salmon meals per week. Controlled feeding trials of other *n*-3 fatty acids have shown them to have positive effects on bone markers.[51] Animal studies also have documented positive effects of fish oil on bone in mice.[52]

Although the correlation between urinary NTX and fish intake data from the long-duration flight study was not as high as the correlation between these variables from the bed rest study, several differences could explain this, two of them being critical to the explanation. First of all, there was a difference in the timing of collections. The bed rest urine data were collected during bed rest, whereas the postflight urine samples were collected after landing and likely do not reflect the level of resorption that would have been seen days earlier during weightlessness. Furthermore, the methods of dietary intake assessment in the two studies were very different. The bed rest dietary intake data were generated from weighing the food items and determining nutrient content from the NDS-R nutrient database, whereas flight dietary intake data came from the answers to a food frequency questionnaire in which food items are grouped into categories based on their content of specific nutrients so that fish with different EPA contents are often combined. The flight data are inherently less accurate for omega-3 fatty acid intake than are the bed rest data, and they should be taken as rough estimates. Nonetheless, the flight data support the bed rest data, and both support the cell culture data showing that EPA inhibited osteoclastogenesis. Although the mechanistic studies support the hypothesis that EPA intake is inhibiting osteoclastogenesis, it is also possible that while consuming fish, these crewmembers/subjects were not consuming other foods that may have been supportive (or passive) with respect to bone resorption.

We looked at the relationship between fish or EPA and bone, but extensive data exist regarding the effects of EPA (and other *n*-3 fatty acids) on other systems of concern for space travelers, including muscle atrophy and immune function. NF-κB activation is associated with several etiologies of muscle protein loss, including disuse. Disuse atrophy (such as occurs in bed rest) is accompanied by activation and increased mRNA expression of NF-κB.[4,6] This mechanism also appears in muscle loss in cancer cachexia, sepsis, diabetes, and other muscle-wasting diseases.[53] The ability of EPA to mitigate the muscle loss in these conditions also has been documented[54,55] and provides strong evidence for the likelihood of success with using it during spaceflight.[53]

The results presented here provide evidence that EPA can decrease NF-κB activation by known activators and modeled weightlessness in a HARV, in addition to decreasing osteoclast differentiation. This evidence is supported in the bed rest and spaceflight models, where bone loss is a known health issue. We now have evidence that NF-κB is activated after short-duration spaceflight, and therefore, inhibition of NF-κB activation could have many beneficial downstream effects to counteract the negative effects of spaceflight on bone, muscle, and immune

function. Beyond muscle, bone, and immune function, the role of *n*-3 fatty acids in cancer prevention is currently being investigated in animal models of spaceflight radiation effects with positive results.[56–61] Thus there is a good possibility that something as simple as a menu change to increase fish intake might serve as a countermeasure to help mitigate risks related to bone, muscle, immune function, and potentially even radiation. Intervention studies with dietary sources of omega-3 fatty acids are warranted to better understand the mechanism of their action on bone and to determine their effects on other physiologic systems (such as muscle and antioxidant defenses). These data will have significant implications for future space exploration and could benefit the general population.

Disclosures

All the authors state that they have no conflicts of interest.

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References

1. Sibonga JD, Cavanagh PR, Lang TF, et al. Adaptation of the skeletal system during long-duration spaceflight. Clin Rev Bone Miner Metab. 2008;5:249–261.
2. LeBlanc AD, Spector ER, Evans HJ, Sibonga JD. Skeletal responses to space flight and the bed rest analog: a review. J Musculoskelet Neuronal Interact. 2007;7:33–47.
3. Trappe S, Costill D, Gallagher P, et al. Exercise in space: human skeletal muscle after 6 months aboard the International Space Station. J Appl Physiol. 2009;106:1159–1168.
4. Durham WJ, Li YP, Gerken E, et al. Fatiguing exercise reduces DNA binding activity of NF-κB in skeletal muscle nuclei. J Appl Physiol. 2004;97:1740–1745.
5. Judge AR, Koncarevic A, Hunter RB, Liou HC, Jackman RW, Kandarian SC. Role for IκBα, but not c-Rel, in skeletal muscle atrophy. Am J Physiol Cell Physiol. 2007;292:C372–382.
6. Hunter RB, Stevenson E, Koncarevic A, Mitchell-Felton H, Essig DA, Kandarian SC. Activation of an alternative NF-κB pathway in skeletal muscle during disuse atrophy. FASEB J. 2002;16:529–538.
7. Farid M, Reid MB, Li YP, Gerken E, Durham WJ. Effects of dietary curcumin or N-acetylcysteine on NF-κB activity and contractile performance in ambulatory and unloaded murine soleus. Nutr Metab. 2005;2:20–28.
8. Rahman MM, Bhattacharya A, Fernandes G. Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. J Cell Physiol. 2008;214:201–209.
9. Fernandes G, Bhattacharya A, Rahman M, Zaman K, Banu J. Effects of *n*-3 fatty acids on autoimmunity and osteoporosis. Front Biosci. 2008;13:4015–4020.
10. Galliera E, Locati M, Mantovani A, Corsi MM. Chemokines and bone remodeling. Int J Immunopathol Pharmacol. 2008;21:485–491.
11. Tilg H, Moschen AR, Kaser A, Pines A, Dotan I. Gut, inflammation and osteoporosis: basic and clinical concepts. Gut. 2008;57:684–694.
12. Whitehouse AS, Smith HJ, Drake JL, Tisdale MJ. Mechanism of attenuation of skeletal muscle protein catabolism in cancer cachexia by eicosapentaenoic acid. Cancer Res. 2001;61:3604–3609.
13. Wigmore SJ, Barber MD, Ross JA, Tisdale MJ, Fearon KC. Effect of oral eicosapentaenoic acid on weight loss in patients with pancreatic cancer. Nutr Cancer. 2000;36:177–184.
14. Riediger ND, Othman RA, Suh M, Moghadasian MH. A systemic review of the roles of *n*-3 fatty acids in health and disease. J Am Diet Assoc. 2009;109:668–679.
15. Smith HJ, Lorite MJ, Tisdale MJ. Effect of a cancer cachectic factor on protein synthesis/degradation in murine C2C12 myoblasts: modulation by eicosapentaenoic acid. Cancer Res. 1999;59:5507–5513.
16. Alexander JW. Immunonutrition: the role of omega-3 fatty acids. Nutrition. 1998;14:627–633.
17. Sun D, Krishnan A, Zaman K, Lawrence R, Bhattacharya A, Fernandes G. Dietary *n*-3 fatty acids decrease osteoclastogenesis and loss of bone mass in ovariectomized mice. J Bone Miner Res. 2003;18:1206–1216.
18. Fernandes G, Lawrence R, Sun D. Protective role of *n*-3 lipids and soy protein in osteoporosis. Prostaglandins Leukot Essent Fatty Acids. 2003;68:361–372.
19. Yaqoob P, Calder PC. N-3 polyunsaturated fatty acids and inflammation in the arterial wall. Eur J Med Res. 8: 2003; 337–354.
20. Micallef MA, Munro IA, Garg ML. An inverse relationship between plasma *n*-3 fatty acids and C-reactive protein in healthy individuals. Eur J Clin Nutr (May 19). 2009;1–5.
21. Moon DO, Kim KC, Jin CY, et al. Inhibitory effects of eicosapentaenoic acid on lipopolysaccharide-induced activation in BV2 microglia. Int Immunopharmacol. 2007;7:222–229.
22. Tisdale MJ. The ubiquitin-proteasome pathway as a therapeutic target for muscle wasting. J Support Oncol. 2005;3:209–217.
23. Kim HH, Lee Y, Eun HC, Chung JH. Eicosapentaenoic acid inhibits TNF-α-induced matrix metalloproteinase-9 expression in human keratinocytes, HaCaT cells. Biochem Biophys Res Comm. 2008;368:343–349.
24. Camandola S, Leonarduzzi G, Musso T, et al. Nuclear factor κB is activated by arachidonic acid but not by eicosapentaenoic acid. Biochem Biophys Res Comm. 1996;229:643–647.
25. Rucci N, Migliaccio S, Zani BM, Taranta A, Teti A. Characterization of the osteoblast-like cell phenotype under microgravity conditions in the NASA-approved Rotating Wall Vessel bioreactor (RWV). J Cell Biochem. 2002;85:167–179.
26. Molnar G, Schroedl NA, Gonda SR, Hartzell CR. Skeletal muscle satellite cells cultured in simulated microgravity. In Vitro Cell Dev Biol Anim. 1997;33:386–391.
27. Pavy-Le Traon A, Heer M, Narici MV, Rittweger J, Vernikos J. From space to earth: advances in human physiology from 20 years of bed rest studies. (1986–2006). Eur J Appl Physiol. 2007;101:143–194.
28. Markwell MA, Haas SM, Bieber LL, Tolbert NE. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. Anal Biochem. 1978;87:206–210.
29. Zwart SR, Lewis BJ. Optimization of detection and quantification of proteins on membranes in very high and very low abundance using avidin and streptavidin. Methods Mol Biol. 2008;418:25–34.

30. Mehta SK, Kaur I, Grimm EA, Smid C, Feeback DL, Pierson DL. Decreased non-MHC-restricted (CD56+) killer cell cytotoxicity after spaceflight. J Appl Physiol. 2001;91:1814–1818.
31. Stowe RP, Sams CF, Mehta SK, et al. Leukocyte subsets and neutrophil function after short-term spaceflight. J Leuk Biol. 1999;65:179–186.
32. Bharti AC, Donato N, Aggarwal BB. Curcumin (diferuloylmethane) inhibits constitutive and IL-6-inducible STAT3 phosphorylation in human multiple myeloma cells. J Immunol. 2003;171:3863–3871.
33. Smith SM, Davis-Street JE, Rice BL, Nillen JL, Gillman PL, Block G. Nutritional status assessment in semiclosed environments: ground-based and space flight studies in humans. J Nutr. 2001;131:2053–2061.
34. Smith SM, Zwart SR, Block G, Rice BL, Davis-Street JE. The nutritional status of astronauts is altered after long-term space flight aboard the International Space Station. J Nutr. 2005;135:437–443.
35. Soller BR, Cabrera M, Smith SM, Sutton JP. Smart medical systems with application to nutrition and fitness in space. Nutrition. 2002;18:930–936.
36. Meck JV, Dreyer S, Warren L. Long-duration head-down bed rest: Project overview, vital signs, and fluid balance. Aviat Space Environ Med. 2009;80 (5, Supplement):A1–A8.
37. Zwart SR, Oliver SM, Fesperman JV, et al. Nutritional status assessment before, during, and after long-duration head-down bed rest. Aviat Space Environ Med. 2009;80:A15–A22.
38. Inniss AM, Rice BL, Smith SM. Dietary support of long-duration head-down bed rest. Aviat Space Environ Med. 2009;80:A9–A14.
39. Schakel SF, Sievert YA, Buzzard IM. Sources of data for developing and maintaining a nutrient database. J Am Diet Assoc. 1988;88:1268–1271.
40. Spector ER, Smith SM, Sibonga JD. Skeletal effects of long-duration head-down bed rest. Aviat Space Environ Med. 2009;80:A23–A28.
41. Kang JX, Weylandt KH. Modulation of inflammatory cytokines by omega-3 fatty acids. Subcell Biochem. 2008;49:133–143.
42. Chopra V, Fadl AA, Sha J, Chopra S, Galindo CL, Chopra AK. Alterations in the virulence potential of enteric pathogens and bacterial-host cell interactions under simulated microgravity conditions. J Toxicol Environ Health A. 2006;69:1345–1370.
43. Bakos A, Varkonyi A, Minarovits J, Batkai L. Effect of simulated microgravity on human lymphocytes. J Gravit Physiol. 2001;8:P69–70.
44. Ulbrich C, Westphal K, Baatout S, et al. Effects of basic fibroblast growth factor on endothelial cells under conditions of simulated microgravity. J Cell Biochem. 2008;104:1324–1341.
45. Granet C, Boutahar N, Vico L, Alexandre C, Lafage-Proust MH. MAPK and SRC-kinases control EGR-1 and NF-κB inductions by changes in mechanical environment in osteoblasts. Biochem Biophys Res Commun. 2001;284:622–631.
46. Kwon O, Tranter M, Jones WK, Sankovic JM, Banerjee RK. Differential translocation of nuclear factor-κB in a cardiac muscle cell line under gravitational changes. J Biomech Eng. 2009;131:064503–064507.
47. Wise KC, Manna SK, Yamauchi K, et al. Activation of nuclear transcription factor-κB in mouse brain induced by a simulated microgravity environment. In Vitro Cell Dev Biol Anim. 2005;41:118–123.
48. Bar-Shai M, Carmeli E, Reznick AZ. The role of NF-κB in protein breakdown in immobilization, aging, and exercise: from basic processes to promotion of health. Ann N Y Acad Sci. 2005;1057:431–447.
49. Visioli F, Rise P, Barassi MC, Marangoni F, Galli C. Dietary intake of fish vs. formulations leads to higher plasma concentrations of *n*-3 fatty acids. Lipids. 2003;38:415–418.
50. Rousseau JH, Kleppinger A, Kenny AM. Self-reported dietary intake of omega-3 fatty acids and association with bone and lower extremity function. J Am Geriatr Soc. 2009; 57: 1781–1788.
51. Griel AE, Kris-Etherton PM, Hilpert KF, Zhao G, West SG, Corwin RL. An increase in dietary *n*-3 fatty acids decreases a marker of bone resorption in humans. Nutr J. 2007;6:2–10.
52. Bhattacharya A, Rahman M, Sun D, Fernandes G. Effect of fish oil on bone mineral density in aging C57BL/6 female mice. J Nutr Biochem. 2007;18:372–379.
53. Tisdale MJ. Mechanisms of cancer cachexia. Physiol Rev. 2009;89:381–410.
54. Fearon KC, Barber MD, Moses AG, et al. Double-blind, placebo-controlled, randomized study of eicosapentaenoic acid diester in patients with cancer cachexia. J Clin Oncol. 2006;24:3401–3407.
55. Khal J, Tisdale MJ. Downregulation of muscle protein degradation in sepsis by eicosapentaenoic acid (EPA). Biochem Biophys Res Commun. 2008;375:238–240.
56. Turner ND, Braby LA, Ford J, Lupton JR. Opportunities for nutritional amelioration of radiation-induced cellular damage. Nutrition. 2002;18:904–912.
57. Davidson LA, Nguyen DV, Hokanson RM, et al. Chemopreventive *n*-3 polyunsaturated fatty acids reprogram genetic signatures during colon cancer initiation and progression in the rat. Cancer Res. 2004;64:6797–6804.
58. Chapkin RS, Davidson LA, Ly L, Weeks BR, Lupton JR, McMurray DN. Immunomodulatory effects of (*n*-3) fatty acids: putative link to inflammation and colon cancer. J Nutr. 2007;137:200S–204S.
59. Hong MY, Bancroft LK, Turner ND, et al. Fish oil decreases oxidative DNA damage by enhancing apoptosis in rat colon. Nutr Cancer. 2005;52:166–175.
60. Sanders LM, Henderson CE, Hong MY, et al. An increase in reactive oxygen species by dietary fish oil coupled with the attenuation of antioxidant defenses by dietary pectin enhances rat colonocyte apoptosis. J Nutr. 2004;134:3233–3238.
61. Vanamala J, Glagolenko A, Yang P, et al. Dietary fish oil and pectin enhance colonocyte apoptosis in part through suppression of PPARδ/PGE2 and elevation of PGE3. Carcinogenesis. 2008;29:790–796.